AD_____

Award Number: DAMD17-97-1-7047

TITLE: Antibody Directed Gene Therapy for Breast Cancer

PRINCIPAL INVESTIGATOR: Peter J. Curtis, Ph.D.

CONTRACTING ORGANIZATION: The Thomas Jefferson University
Philadelphia, Pennsylvania 19107-6799

REPORT DATE: February 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Final (1 Jul 97 - 1 Jan 01)

4. TITLE AND SUBTITLE
Antibody Directed Gene Therapy for Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7047

6. AUTHOR(S)
Peter J. Curtis, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Thomas Jefferson University
Philadelphia, Pennsylvania 19107-6799

E-Mail: pcurtis@lac.jci.tju.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The main objective of this proposal is to develop monoclonal antibodies as cell specific targeting vectors so that they are able to bind and carry DNA into cells. The molecularly engineered antibody targeted DNA to tumor cells, but the expression of the DNA was low. Different strategies have been examined to improve the expression. These strategies have included incorporation of a flu fusion peptide into the antibody/DNA complex, DNA condensation, and the role of viral origins of replication in the transport of naked DNA into the nucleus. The major barrier remained the cytoplasmic membrane.

To achieve entry into the cytoplasm, the entire domain containing the flu fusion peptide, HA2, was engineered to contain a DNA binding domain, and expressed by the baculovirus/insect cell system. The fused HA2 protein, upon purification, was shown to have the signal peptide still attached. Thus, the HA2 did not have glycine as its N-terminal amino acid, which is essential for its fusogenic property. Thrombin and factor Xa recognition sites were incorporated into the N-terminus of HA2 to provide a way to get a N-terminus glycine in the HA2.

14. SUBJECT TERMS
Breast cancer, gene therapy, bispecific antibody, nonviral vector, growth factor receptors

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Breast cancer, like most cancers, develops as a result of several mutational events. These events result in tumor cells capable of considerable phenotypic variability, so that within a large population there are cells that are able to evade the immune system and frequently are capable of spreading to distant tissues to form metastatic lesions. Yet patients do produce some immune response to their tumors as indicated by recovery of tumor specific cytotoxic lymphocytes and occasionally spontaneous regression. The strategy of transfecting cytokine genes into tumor cells to enhance the host immunity has been extensively studied in animal models and has provided the groundwork for human clinical trials. However these approaches rely on ex vivo modification of tumor cells, which is difficult, time consuming, expensive and dependent on research facilities. Vectors capable of targeting a particular cell type, i.e tumor cells, are urgently required to achieve tumor cell modification in vivo.

In this proposal we intend to develop a breast tumor-specific targeting vector by combining two different monoclonal antibodies, mAb425, which binds human epidermal growth factor receptor(EGFR) and Her66 and 81, which bind to human erbB2, both of which are growth factor receptors abundantly expressed on a subset of breast tumors. One arm of this bispecific antibody will be further modified by molecular engineering to contain DNA encoding a DNA binding domain into the gene encoding the heavy chain, so that the bispecific antibody will be capable of binding DNA. A number of strategies, designed to optimise entry into the cytoplasm, transport to the nucleus and expression for a prolonged period of time, will be combined to produce a novel breast tumor specific targeting vector capable of delivering DNA into tumor cells.

Body

Statement of Work

Technical Objective 1

Task 1. Clone, sequence and express Her81 as a Fab.
Task 2. Produce 425fosLZ/Her81junLZ bAb and study its binding to tumor and normal cells.

Technical Objective 2 and 3

Task 3. Produce 425LZ.DBD and transfect human breast cell lines.
Task 4. Optimize expression of DNA by:

a) addition of flu fusion peptide
b) addition of nuclear localization signal.
c) selection of replicating plasmid.
d) analysis of putative tumor specific promoters.

Technical Objective 4

Task 5. Clone CMV-b-galactosidase gene into replicating plasmid.
Task 6. Produce 425fosLZ.DBD/Her81junLZbAb complexed with CMV-b-galactosidase plasmid.
Task 7. Study targeting of human breast tumor cells in nude mice.

Task 1. Clone, sequence and express Her81 as a Fab.

Since cloning and expression of a Fab can encounter unexpected difficulties we obtained a second hybridoma, Her66, which also binds erbB2, from Dr. E. Vitetta. RNA prepared from the hybridomas Her66 and 81, was used as template to clone the light and heavy chain cDNAs by RT-PCR using oligonucleotide primers, which introduce restriction sites to facilitate subcloning into

pFab. pFab is a derivative of pUC19 with the multicloning site replaced by restriction sites suitable for the cloning and assembly of IgG cDNAs. The light and heavy chains of Her66 and 81 have been cloned and sequenced.

Task 2. Produce 425fosLZ/Her81junLZ bAb and study its binding to tumor

The light and heavy chains of Her66 and Her81 have not been subcloned yet into the baculovirus transfer vector and have not been expressed.

Task 3. Produce 425LZ.DBD and transfect human breast cell lines

DNA encoding the DNA binding domain(DBD) of histone H1 was cloned into the C terminal codon of 425/fosLZ cDNA and then transferred into a recombinant baculovirus(rBEV). Co-infection of insect cells with rBEV.425K with rBEV.425/fos/DBD resulted in a 425 Fab with a DBD tail. The production and purification of 425fos/DBD has been improved considerably from the initial submission of this proposal.

The amount of 425fos/DBD found in the supernatant of infected cells was surprisingly low. It was realised that 425fos/DBD contains many positive charges, so that the secreted DBD might bind to the surface of the infected insect cells, which has a net negative charge. Washing infected cells with 1.0M NaCl released much more 425fos/DBD than found in the supernatant. Since the cell surface is limited and bound 425fos/DBD might be internalised, addition of negatively charged beads might increase the yield of 425fos/DBD. Further improvement was made by noting that maximal expression of 425fos/DBD was achieved only when an excess of 425 kappa chains was produced, but the excess kappa chains saturate the anti-mouse kappa affinity column. Therefore an ion exchange column was used prior to the affinity column to eliminate the excess kappa chains.

The procedure now used is as follows. Twenty four hours post infection, a slurry of sephadex SP-50, equilibrated with the insect medium, Express 5,is added to the infected cells, and the flasks are gently rocked to ensure mixing of the beads with the cells. After 50-60 hours the cells and beads are harvested by centrifugation, the pellet suspended in 0.15M NaCl, 10mM Tris pH 7.5, 10mM EDTA, 100ug/ml PMSF and then adjusted to 1.0M NaCl. Cells and beads are removed by centrifugation. The supernatant, after being diluted to 0.3M is passed over a sephadex SP-50 column, which is washed with 0.3M NaCl and tris, EDTA and PMSF, followed by 1.0M NaCl to elute 425fos/DBD, with very little 425 kappa chain.

In the next step the 1.0M eluant is applied undiluted to the anti-mouse kappa affinity column. The reason for applying 425fos/DBD in 1.0M NaCl is because molecules capable of inhibiting the binding of DNA by 425fos/DBD, possibly sulphated mucopolysaccharides, are eluted from the insect cells by 1.0M NaCl; any polyanions will associate with the 425fos/DBD at low salt. After washing with 1.0M NaCl the column is washed with a low salt buffer, followed by elution by a neutral buffer(buffer C, provided with the column by Sterogene, Calif). This step eliminates any DBD cleaved from the Ab, as well as any inhibitory polyanions. The eluting buffer is diluted to less than 0.5M salt before applying to a HiTrap SP column(Pharmacia). The 425fos/DBD is eluted by 1.0M NaCl followed by dialysis against PBS.

From 6 T175 flasks seeded with 30 x 106 cells per flask, about 430 ug purified 425fos/DBD was recovered, as estimated by OD280 and by comparison with known amounts of mAb17-1A on a SDS gel stained with coomassie blue(Fig. 1).

The ability of 425Fab.DBD to bind DNA was demonstrated by gel electrophoresis of different ratios of 425Fab.DBD to plasmid(Fig. 2).

The specificity of 425fos/DBD binding was demonstrated by FACS analysis, using human A431 cells and mouse CT26 cells which do not bind mAb425. No significant binding to the CT26 cells was detected by the Ab alone or complexed with DNA(Table 1).

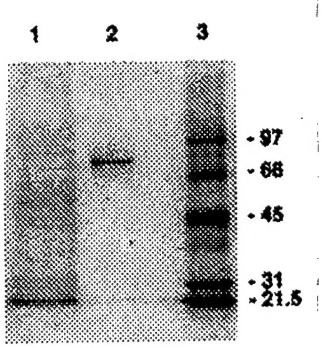


Figure 1. SDS PAGE of 425Fab.DBD. Lane 1, Cells and slurry 1.0M wash, 20ul. Lane 2, purified 425Fab.DBD, 5ul. Lane 3, Protein size markers. Stained with Coomassie Blue.

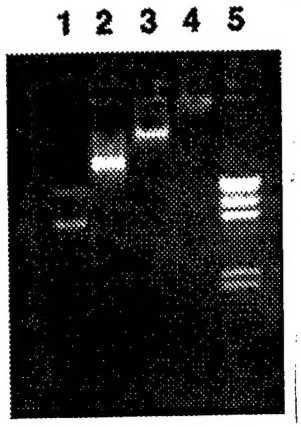


Figure 2. Agarose gel of 425Fab.DBD/DNA. Lane 1, pCMVLuc. Lane 2, Ab/DNA ratio 0.8:1.Lane 3, 1.7: 1. Lane 4, 3.3:1. Lane 5, _HindIII size marker

Table 1

		Mean Fluorescence Intensity	
425DBD ug/ml	DNA ug/ml	CT26 Cells	A431 Cells
0	0	3.9	5.7
1.4	0	3.9	-
4.6	0	4.3	-
14.0	0	4.8	-
1.4	1.8	4.2	160.3
4.6	5.5	4.1	249.7
14.0	16.6	4.2	166.9

These complexes were added to human A431 cells, which express EGF-R abundantly, and 983-B cells, which express a much lower level of EGF-R, at different concentrations at 37°C for 4 hours in the presence of chloroquine(100uM) followed by 24 hours in medium alone, after which the cells were trypsinized, washed, lysed and assayed for luciferase. The results showed that the Ab/DBD concentration must be at least 4 ug/ml, and complexes with a higher ratio of Ab/DNA resulted in more luciferase. In experiment 2, DNA was reduced by a factor of 4 with Ab/DBD kept constant without significantly diminishing luciferase activity(Table 2).

Table 2

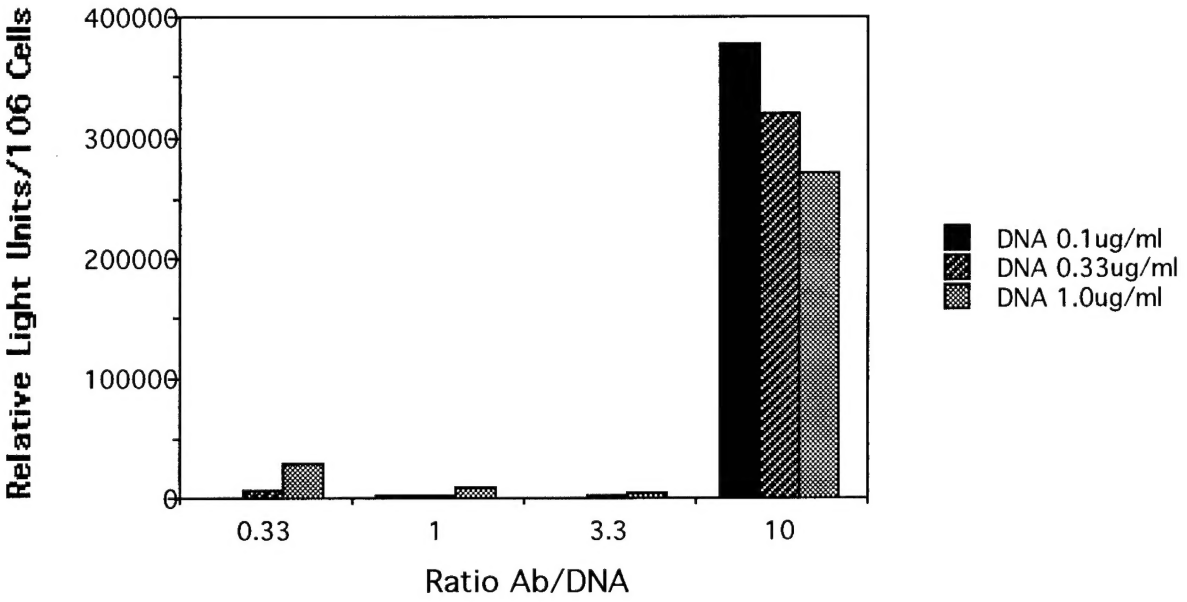
	Ab : DNA (ug/ml)	RLU/106 cells
Expt. 1-A431 cells	0.8 : 6.0	0
	0.8 : 1.2	0
	4.0 : 30.0	0
	4.0 : 6.0	3120
983-B cells(-EGF-R)	4.0 : 6.0	220
Expt. 2	6.0 : 7.2	10,720
	6.0 : 3.6	10,720
	6.0 : 1.8	10,360

Task 4. . Optimize expression of DNA . a) addition of flu fusion peptide

Further experiments were performed to identify the conditions to achieve optimal transfection. 425fos/DBD was mixed with DNA at different ratios, diluted upto 10 fold, and added to human A431 cells in the presence of chloroquine(100uM). A very high level of luciferase was detected using

a ratio of Ab/DNA of 10. It was not concentration dependent over the range tested, as the same result was obtained with a concentration of 1.0ug/ml Ab as well as 10ug/ml(Fig. 3).

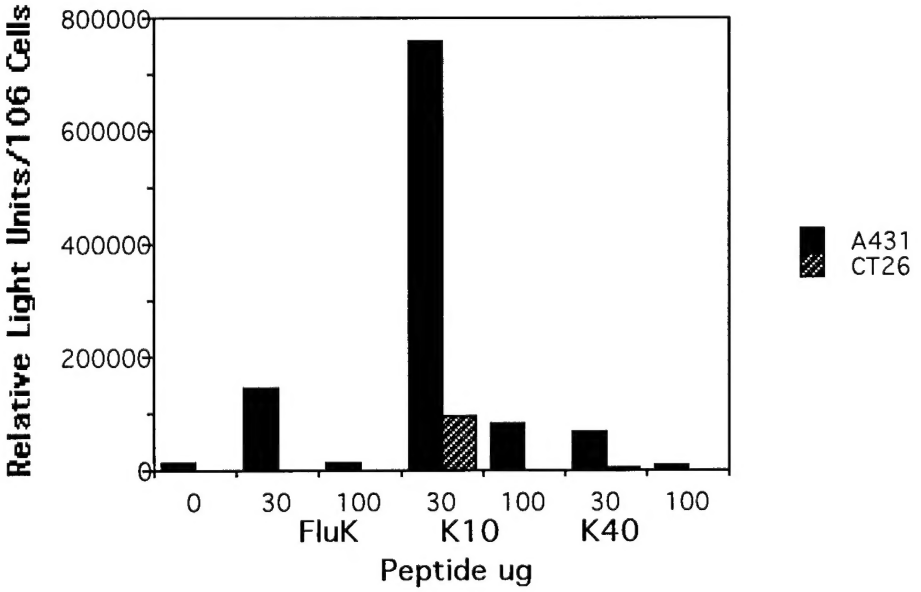
Figure 3.



This result suggested that covering the DNA negative charges may be important to achieve efficient transfection. If this is the explanation, it should be possible to substitute polylysine for some of 425fos/DBD, which will also reduce the size of the complex. To avoid crosslinking to more than one DNA molecule, short polylysines were used, K10-10 lysines, andK40-40 lysines. In addition, the flu fusion peptide was synthesized with a C-terminal tail (KKKP)3. Each peptide bound DNA, as judged by gel mobility shift on an agarose gel, and these complexes were stable to prolonged dialysis.

For this experiment, the Ab was mixed with DNA at a ratio of 2.2:1. It was found that only concentrations of the peptides much higher than needed to cover the DNA negative charge were effective. The highest level of luciferase was obtained using 0.15mg/ml K10(Fig 4). Under these conditions the complex still targeted specificially human A431 cells. However the flu peptide did not improve luciferase expression.

Figure 4.



.The above results did not show linearity or proportionality of the response to varying the Ab/DNA complex. An explanation for this nonlinearity could be that DNA condensation occurs only under specific conditions. It has been shown that DNA condensation increases the efficiency of DNA uptake and expression(1, 2). DNA, polylysine and salt concentrations are critical to achieve DNA condensation.

DNA condensation results in the formation of a toroid, about 50 nm in diameter, which with time forms larger aggregates. Attempts were made to identify conditions for DNA condensation using 425Fab.DBD, complexed with DNA, by electron microscopy, but viewing of grids showed always a variety of structures in addition to objects that had the dimensions expected for condensed DNA.

A simpler way of assessing DNA condensation is filtration through a 0.2um filter, combined with testing for DNAse I resistance, a property of condensed DNA. Initially, polylysine with 10, 40 and 180 residues was added to DNA at 0.15 M NaCl, but the resulting complexes appeared to stick to plastic tubes. This was not a problem using the Ab/DNA complex. Polylysine , average length 10, 40 and 180 was added to the Ab/DNA complex at different concentrations either in one or many steps over different periods of time. The Ab/DNA complex was used at low concentrations on the assumption that its lower concentration would tend to minimize the process of aggregation. It was found that optimal conditions for DNA condensation and DNAse I resistance was achieved using the Ab/DNA complex at 0.5 ug/ml and polylysine, average length 10, added to a final concentration of 6 ug/ml over a period of 1 hour at 37^0C(figure 5). The Ab/DNA /polylysine complex was stable for 3-4 hours before aggregation occurred.

However the use of such complexes on the target cells, human A431, did not result in any improvement in the expression of the DNA. Presumably other barriers to the expression of the DNA must exist.

To identify the fate of the bound complex, a comparison was made between complexes assembled with 425fos/DBD and with Superfect(an oligodendrimer, Qiagen) upon transfection of human A431 cells. The 425fos/DBD complex was bound to cells at 4^0C for 2 hours, so that the amount bound could be estimated in the absence of internalization and degradation. After 2 hours at 4^0C, one plate was used to isolate bound plasmid, while two plates were shifted to 37^0C for 3 and 20 hours,at which time cells were trypsinized and internalized plasmid was isolated by the alkaline lysis method. Plasmid DNA was estimated by Southern blot in comparison with known amounts ofplasmid. The Superfect/DNA complex was placed on the cells at 37^0C for 45 minutes. Plasmid was isolated from trypsinized cells incubated for a further 3 and 20 hours, as above(Table 3).

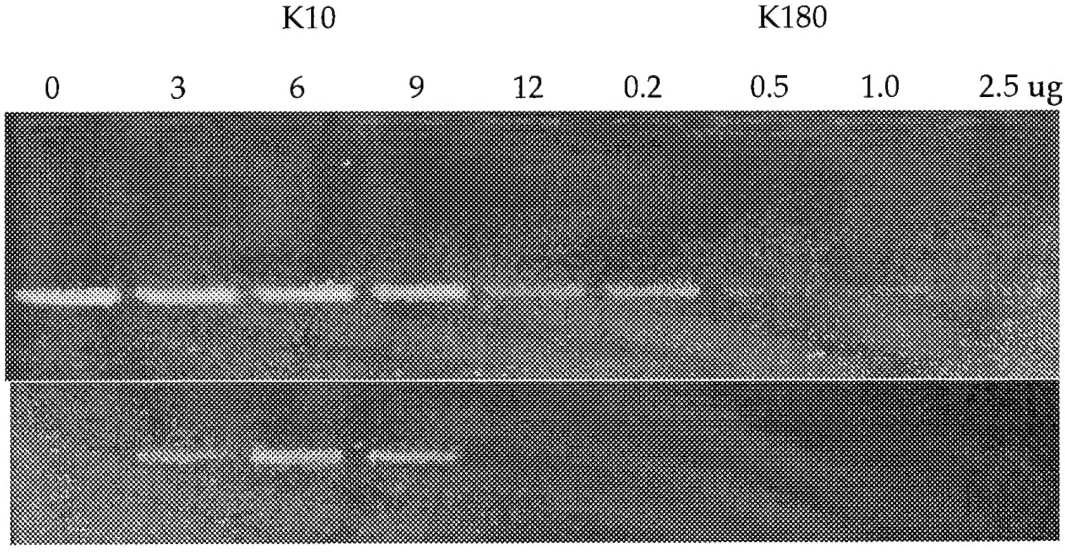


Figure 5. Agarose gel electrophoresis of DNA recovered from Ab/DNA complexes with polylysine-10 residues(K10), or 180 residues(K180) after filtration. Lower panel, DNA recovered after filtration and DNAse I.

Table 3.

	Estimated DNA ug/10^6 cells			
Complex	4^0C	3 hrs	20 hrs	RLU/10^6 cells
425fos/DBD	0.3 (10%)	0.03 (1%)	0.005 (0.15%)	10^5
Superfect	-	0.3 (3%)	0.3 (3%)	1-5x10^7

For the 425fos/DBD complex upto 10% of the input DNA was bound to the cells, but most of the DNA was lost after 20 hours. The same result was obtained using a complex of 425fos/DBD and DNA mixed with polylysine under conditions where most of the complex is filterable. With Superfect, DNA appeared to be more stable inside the cell, at least upto 20 hours. The amount found after 20 hours corresponds to about 60,000 DNA molecules/cell, yet if human A431 cells were transfected in the same way with a β-galactosidase plasmid, less than 10% of the cells were stained. This result suggests that very few DNA molecules are actively transcribed, probably because they do not enter the nucleus.

The major barriers that the DNA must cross to achieve expression are the cytoplasmic and nuclear membranes. The influenza hemagglutinin(HA) has been shown to be the only protein necessary for entry of the viral particle into the cytoplasm. It is a very efficient fusogen, which has been extensively studied. It contains two domains, HA1 which is the binding domain for sialic acid residues, and HA2, whose N terminus initiates fusion. The X-ray structure of HA1 and 2 have been determined not only at neutral pH but also at acid pH where extensive rearrangement of their structure occurs which facilitates fusion of the viral membrane with the cytoplasmic membrane(3). At the acid pH, HA1 does not undergo any major change, but HA2 changes dramatically, so that its N-terminal fusion domain projects outward at the end of a long coiled coil. The N-terminus inserts into the cytoplasmic membrane, while its C-terminus including the transmembrane domain moves closer to the N-terminus by a fold back mechanism, thus bringing the two membranes close together(4,5). The other domains of HA2 in addition to the fusion peptide clearly play an important role increasing the efficiency of fusion

The DNA encoding the HA2 domain lacking its transmembrane and cytoplasmic domains has been cloned by PCR and joined with a signal peptide derived from mouse kappa cDNA, which has been used for the expression of several proteins in the baculovirus/insect cell system. The cloning was accomplished by use of overlapping PCR to achieve fusion of HA2 DNA to that of the signal peptide and introducing alanine residues to the -3 and -1 positions of the signal peptide to conform with the most frequently observed residues at the signal peptide cleavage site(6). It was found that it was not possible to obtain stable clones in pUC19 , but possible in pUC 8. This was presumably because leaky expression of the HA2 peptide occured from the lac promoter in pUC 19 and expression of the HA2 peptide is lethal. Next the DNA encoding the DNA binding domain(DBD) was added to the HA2 DNA.

Several different approaches were tried to to subclone the HA2.DBD into the transfer vector, pVL1393. It was finally achieved by cloning the HA2.DBD as two separate restriction fragments into pVL1393, from which recombinant baculovirus was made. Infection of insect cells with the recombinant baculovirus produced HA2.DBD polypeptide, detected by Western blot analysis using a HA2-specific monoclonal antibody(kindly provided by Dr. Walter Gerhardt, the Wistar Institute). The fused HA2.DBD was harvested by washing the cells and SP-Sephadex(added to the cells 24 hours after infection, as described earlier in the production of 425fos.DBD) with 1M sodium chloride.

The eluted protein was found to be insoluble. It was possible to dissolve it in 8M urea and purify the HA2.DBD by passage over a HiTrap-SP column in 0.3M sodium chloride in 8M urea, followed by elution with 1M sodium chloride in 8M urea. SDS gel analysis showed a single band. Amino acid sequence analysis showed that the mouse kappa signal peptide had not been cleaved from the HA2.DBD, even though the C-terminal sequence of the signal peptide had been altered to the most frequently observed amino acids at the signal peptide cleavage site.

Since the presence of a N-terminal glycine is essential for the fusing activity of HA2, oligonucleotides encoding the recognition sequences for the proteases factor Xa and thrombin were synthesized. The recognition sequence of factor Xa is I-E-G-R/X, which is not present in HA2.DBD. While thrombin is less specific, it has been used to cleave several fused proteins and is much cheaper than factor Xa. The double stranded oligonucleotides were ligated between the mouse kappa signal sequence and the HA2.DBD using a restriction site, NgoAIV, that had been introduced in the construction of the plasmid. The constructs containing the factor Xa and thrombin cleavage sites are being introduced into the transfer vector, pVL1393, prior to producing recombinant baculovirus and testing which of the two proteases will cleave the signal peptide to generate a N-terminal glycine.

Task 4. Optimize expression of DNA by: b) addition of nuclear localization signal.

For efficient expression of transfected DNA nuclear localization is important. Though DNA transfected into cells in various ways is expressed, the efficiency is very variable, and probably largely dependent on DNA gaining access to the nucleus during cell division, when the nuclear membrane disassembles. In general transfection efficiency is known to depend upon cells plated at less than 100% confluency so that they can continue to divide. DNA microinjected into the cytoplasm is not expressed(7).

The nuclear localization signal(NLS) has been well defined in SV40 proteins, large T antigen, Vp2 and Vp3, and consists of the sequence PKKKRKV. NLS peptides have been linked to DNA in different ways with variable results. In one study, a single NLS peptide was covalently linked to a plasmid, and resulted in a dramatic increase in the efficiency of expression(8). The authors suggested that DNA containing multiple NLS might become bound by more than one pore. In another study, at least 101 NLS peptides/1kbp of DNA was necessary to achieve nuclear transport(9). In this case perhaps the NLS peptides, because of its positive charges, interact electrostatically with the DNA phosphate backbone and are mainly unavailable to bind to _-importin.

DNA encoding NLS has been fused to the C-terminus of mouse gamma signal peptide and to the N-terminus of c-fos LZ linked to DBD. It was expressed by infection with a recombinant baculovirus of insect cells, harvested by elution from the cells and SP-Sephadex by 1M sodium chloride, and purified by passage over a HiTrap-SP column. SDS gel analysis showed a single band. Amino acid sequence analysis revealed that a cleavage had occurred within the signal peptide leaving the NLS attached to the DBD.

The presence of c-fos LZ between NLS and DBD should prevent the NLS from binding to DNA and being inaccessible. The c-fos LZ has an excess negative charge, as well as hydrophobic residues and would be expected to be repelled by the negative charge of the DNA.

A few studies have shown that naked DNA is not transported into the nucleus. However naked SV40 DNA, but not pBR322, is imported into the nucleus via α-importin and dependent on transcription(10). The SV40 sequences necessary for importing were the origin of replication, parts of the early and late promoters and the enhancer. A 100 fold increase of gene expression was also obtained by adding Epstein-Barr viral origin of replication as well as EBNA1(11).

To check on the role of DNA containing origins of replication on nuclear transport, four constructs were compared. Each construct contained the luciferase gene driven by the CMV early promoter. In addition, plasmids contained the human BKV origin and T antigen(BKV is very similar to SV40), or the human Epstein-Barr viral origin and its EBNA I, or a 250 bp DNA fragment containing the dyad symmetry element from the Epstein-Barr origin. Equal amounts of each plasmid was transfected using Superfect(Qiagen) into human A431 cells, and after 24 hours, the cells were lysed and assayed for luciferase(Table 4).

The presence of the origins increased the expression by at least 10 fold, and the 250 bp fragment was sufficient. Clearly this small element will be added to plasmids used for transfections. It has been suggested that newly translated transcription factors in the cytoplasm bind to sequences in the DNA origins and as a result the complexes are transported into the nucleus via the NLS in the

transcription factor. It will be interesting to determine if addition of NLS containing peptides to Ab/DNA complexes will further improve their expression.

Table 4.

DNA Sequences	RLU/10^6 cells
-	2.2×10^7
BKV ori + T antigen	3.0×10^8
E-B ori + EBNA I	4.5×10^8
E-B dyad symmetry element	3.4×10^8

Task 4. Optimize expression of DNA by: c) selection of replicating plasmid.

In order to achieve prolonged expression of transfected DNA, plasmids have been constructed that should be episomally replicated in different human tumor cells. The BKV origin of replication was cloned into pUC19 together with the BKV T antigen gene driven by a CMV promoter. Different orientations were identified. To detect replication, plasmids were transfected by DEAE-dextran into a human adenocarcinoma HT29 cells. After 3 days, Hirt supernatants were prepared, digested with Dpn I, to destroy plasmid methylated in E.coli., i.e. unreplicated DNA, and transfected into E.coli. The results of two experiments are shown in Table 5. As expected the T antigen has to be present to obtain replication in the human cells.

Table 5.

	Ori T ag	Total no. colonies
1	->	0 , 0
2	<-	0 , 0
3	-> ->	1150 , 1690
4	-> <-	2760 , 5600
5	<- ->	2490 , 980
6	<- <-	2050 , 2760

Transfection of SV40-based plasmids into COS cells, expressing SV40 T antigen results in plasmid replication upto 50,000 copies per cell over a period of 3-4 days, but expression is lost after this time, possibly due to selection against cells containing large numbers of plasmids.

Mutagenesis was performed on the BKV origin of replication in order to select a BKV-based plasmid that would be capable of being maintained for a long period of time in human HT29 cells. The BKV origin of replication was amplified by PCR in the presence of dITP to promote random mutagenesis. The PCR fragment was cloned and individual clones were sequenced. On average each clone contained 2-3 base changes. Plasmid was prepared from about 10,000 colonies. The fragment containing the origin of replication was excised and cloned into pUC19 containing BKV T antigen, driven by a CMV promoter. Plasmid , prepared from 30,000 colonies was transfected into human HT29 cells. After 2 and 3 weeks, plasmid was extracted from the HT29 cells, digested with DpnI and transfected into E.coli. Fifty six colonies were obtained from plasmid harvested after 2 weeks and 27 colonies after 3 weeks. Plasmid, prepared from the pools of colonies as well as the unselected pool was transfected into human HT29 cells. Plasmid was harvested from the cells after 1, 2 and 3 weeks, and transfected into E.coli. before and after digestion with DpnI(Table 6).

The results did not show more replicated plasmid from HT29 cells transfected with 2 week or 3 week selected plasmid than with the unselected plasmid pool, so there is no evidence of selection of a plasmid capable of being maintained over several weeks. In all cases however replicated plasmid was found after 2 and even 3 weeks. The other noteworthy result is that even after 2 weeks the majority of plasmid recovered from the HT29 cells has not been replicated, indicating that a majority of the plasmid may not have entered the nucleus, even though the BKV origin of replication was present in the plasmid.

Table 6.

		Number of Colonies		
Plasmid	DpnI	1 Week	2 Week	3 Week
Unselected	-	420	140	20
	+	140	72	4
2 week	-	3000	1750	20
	+	220	60	12
3 week	-	4800	1250	70
	+	448	108	4

Tasks 5, 6 and 7.

These tasks were not accomplished. The reason for this was the failure to achieve efficient transfection of human breast cancer cells by the antibody-DNA complex, which was essential for these tasks to be of value. All the effort has been directed at achieving efficient transfection.

Key Research Accomplishment

a) Monoclonal antibodies that recognize human epidermal growth factor receptor(EGFR) and erbB2 have been cloned and engineered to contain a DNA binding domain(DBD).

b) Anti-human EGFR with a DBD has been expressed abundantly in the baculovirus/insect cell system, from which it has been purified.

c) Anti-human EGFR with a DBD, complex with DNA, delivered the DNA specifically to the target cells, a human adenocarcinoma, which expresses high levels of human EGFR.

d) Strategies are being pursued to increase the efficiency of targeting and expressing the DNA in the human tumor cells.

Reportable Outcomes

- Manuscripts etc. None

- Patents. None.

- Funding applied for:

a). Title – Targeting of a DNA Vaccine – American Cancer Society – rejected.

b). Title – Targeting a DNA Vaccine – NIAID RO3-AI45699 – funded.

c). Title – DNA Targeting of Cancer Cells by an Engineered Antibody – NCI RO1-CA85625-01 – rejected.

Conclusions

The cDNAs for mAb425 have been genetically engineered to contain a DNA binding domain(DBD), so that co-expression of the light and heavy chain cDNAs as recombinant baculoviruses in insect cells results in the production of a Fab with a DBD that bound DNA as well as its target cell, human A431. The Ab/DNA complex specifically targeted human A431 cells resulting in the expression of the reporter gene, luciferase. However more than 99% of the targeted DNA was rapidly degraded, presumably by being internalized into endosomes, which quickly fuse with

lysosomes. Incorporation of the influenza fusion peptide into the Ab/DNA complex did not facilitate entry of the DNA into the cytoplasm. Though DNA condensation had been shown to greatly increase expression of transfected DNA, it was not effective in our system. However there are DNA sequences in viral origins of replication that facilitate transport of DNA into the nucleus. A 250 bp

fragment containing the dyad symmetry element from human Epstein-Barr viral origin was sufficient for this transport. In addition DNA encoding a NLS has been joined to DNA encoding the DBD, so it also can be incorporated into the same complex to facilitate the transport of the DNA into the nucleus.

To achieve more efficient entry of targeted DNA into the cytoplasm, the HA2 domain from the influenza hemagglutinin gene has been engineered so that it can be incorporated into the Ab/DNA complex.

However two problems were encountered. Cleavage of the signal peptide from the HA2 did not occur. This cleavage would have generated a N-terminal glycine on the HA2 molecule, which is essential for the fusing actvity of HA2. The second problem is that the HA2.DBD polypeptide is insoluble.

To overcome the first problem, two protease recognition sites have been introduced between the signal peptide and the HA2.DBD. The modified HA2.DBD, dissolved in 8M urea, will be bound to a Hitrap-SP column. The column will then be washed free of urea, treated with the protease, after which the cleaved HA2.DBD will be eluted from the column in 8M urea and high salt.. The second problem will require identifying conditions to allow HA2.DBD to renature while bound to DNA.

The major problem with cancer is the spread of metastatic lesions. Chemotherapy and radiation are clumsy and inefficient tools to combat these lesions. The ability to target DNA to tumor cells in vivo is an exciting approach. DNA encoding one or more cytokine targeted to tumor cells can induce an immune response to the tumor cells, particularly cytotoxic T cells, and once induced the CTLs would monitor the whole body for remaining tumor cells. Therefore the targeting of DNA to tumor cells in vivo is a very important goal. Viral vectors, such as adenovirus and retroviruses, are not suitable for in vivo use, since they infect a variety of cells. In addition many people have antibodies to adenovirus, which will limit the effectiveness of this vector, and if not adenovirus induces usually a strong immune response. Effects to retarget these viral vectors have so far not been successful.

The approach described in this project is to develop a nonviral vector that will target specific cells as determined by the antibody's specificity. Though there are technically difficult goals to achieve, it is extremely important to have a therapeutic agent that could be injected into a cancer patient, target the DNA to tumor cells to induce an immune reponse to the tumor cells. Such an immune response should be able to seek out any surviving tumor cells.

References

1. Perales, J.C., G.A. Grossmann, M. Molas, G. Liu, T. Ferkol, J. Harpst, H. Oda and R.W. Hanson, Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. Journal of Biological Chemistry, 1997. **272**(11): p. 7398-407.
2. Wagner, E., M. Cotten, R. Foisner and M.L. Birnstiel, Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(10): p. 4255-9.
3. Bullough, P.A., F.M. Hughson, J.J. Skehel and D.C. Wiley, Structure of influenza haemagglutinin at the pH of membrane fusion [see comments]. Nature, 1994. **371**(6492): p. 37-43.
4. Chen, J., K.H. Lee, D.A. Steinhauer, D.J. Stevens, J.J. Skehel and D.C. Wiley, Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell, 1998. **95**(3): p. 409-17.

5. Hughson, F.M., Enveloped viruses: a common mode of membrane fusion? Current Biology, 1997. **7**(9): p. R565-9.
6. Nielsen, H., J. Engelbrecht, S. Brunak and G. von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering, 1997. **10**(1): p. 1-6.
7. Capecchi, M.R., High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell, 1980. **22**(2 Pt 2): p. 479-88.
8. Zanta, M.A., P. Belguise-Valladier and J.P. Behr, Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(1): p. 91-6.

9. Ludtke, J.J., G. Zhang, M.G. Sebestyen, and J.A. Wolff, A nuclear localization signal can enhance both the nuclear transport and expression of 1 Kb DNA. Journal of Cell Science, 1999. 112(12): p2033-41.
10. Dean, D.A., Import of plasmid DNA into the nucleus is sequence specific. Experimental Cell Research, 1997. **230**(2): p. 293-302.
11. Langle-Rouault, F., V. Patzel, A. Benavente, M. Taillez, N. Silvestre, A. Bompard, G. Sczakiel, E. Jacobs and K. Rittner, Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. Journal of Virology, 1998. **72**(7): p. 6181-5.